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RESEARCH ARTICLE

Prevention of restenosis by a herpes simplex virus mutant capable of controlled long-term expression in vascular tissue in vivo

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Neointimal hyperplasia resulting from vascular smooth muscle cell (SMC) proliferation and luminal migration is the major cause of autologous vein graft failure following vascular coronary or peripheral bypass surgery. Strategies to attenuate SMC proliferation by the delivery of oligonucleotides or genes controlling cell division rely on the use of high concentrations of vectors, and require pre-emptive disruption of the endothelial cell layer. We report a genetically engineered herpes simplex virus (HSV-1) mutant that, in an in vivo rabbit model system, infects all vascular layers without prior injury to the endothelium; expresses a reporter gene driven by a viral promoter with high efficiency for at least 4 weeks; exhibits no systemic toxicity; can be eliminated at will by administration of the antiviral drug acyclovir; and significantly reduces SMC proliferation and restenosis in vein grafts in immunocompetent hosts. Gene Therapy (2001) **8**, 1840–1846.

Keywords: *neointimal hyperplasia; vein graft restenosis; herpes simplex virus; gene therapy*

Introduction

Disorders of the vascular system are the leading causes of death and disability in the western world. Currently, over 60 000 000 Americans suffer from cardiovascular disease (American Heart Association; http://www.americanheart.org). Risk factor management including the use of cholesterol-lowering drugs can be effective prevention, but therapy for established lesions requires mechanical intervention, including endovascular (angioplasty, stenting) and surgical revascularization (endarterectomy, bypass grafting). Intervention is almost always initially technically successful, but the vascular response to injury and subsequent restenosis continues to compromise long-term results. Restenosis occurs in 30% of percutaneous coronary angioplasty procedures, 50% of peripheral and coronary vein grafts, and up to 80% of percutaneous procedures performed for complex infrainguinal lesions.[1,2] As the annual cost of mechanical treatment of atherosclerotic lesions exceeds $300 billion, restenosis remains one of the most significant health care problems in this country.

Clinical and basic scientific investigation has revealed that the most common cause of restenosis is neointimal hyperplasia, a pathologic proliferation of vascular smooth muscle cells (SMC) that occurs in response to physical manipulation.[3] When severe, the process leads to the formation of new encroaching lesions within the vessel lumen, reductions in blood flow and perfusion and, ultimately, therapeutic failure. Neointimal hyperplasia has been surprisingly recalcitrant to any form of systemic pharmacologic treatment,[4] leading some investigators to conclude that its prevention might only be achieved locally, possibly using antiproliferative gene therapy.

In the past 10 years, gene therapy strategies have been designed to control neointimal hyperplasia by the delivery of antisense oligonucleotides, cell cycle regulators, and/or noxious viral genes.[5] Popular vectors under investigation include plasmid DNA, liposomes, retroviruses, adenoviruses and adeno-associated viruses. All of these vectors, however, exhibit fairly low penetrance into vascular tissue when the basement membrane is intact.[6–12] Therefore, most strategies have stipulated preemptive mechanical denudation of the endothelium, followed by delivery of very high doses of vector. Even with these modifications, the observed duration of reporter gene expression has been limited, and most of its biological activity is undetectable by the end of the first week.[11,13,14] In addition, delivery of large amounts of vector leads to stimulation of the immune response that results in rapid elimination of virally infected cells. Immunosuppression of the host may extend the life of the exposed cells,[15] but global immunosuppression is impractical for most patients, and particularly for the elderly population with vascular disease.

Genetically modified HSV-1, especially those attenu-

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ated by removal of the $\gamma_1$34.5 genes, have been extensively studied as highly selective oncolytic agents for therapy of malignant tumors of the central nervous system.[16–18] Its predilection for dividing cells is exploited in the present strategy wherein HSV-1 is used to selectively target proliferating vascular SMCs. The strain R3616 lacks both copies of the $\gamma_1$34.5 gene which are required to block the shutoff of protein synthesis caused by the activation of protein kinase R, and this is the basis for the attenuated behavior of the mutant (Figure 1). $\gamma_1$34.5-minus mutants replicate 100- to 1000-fold less efficiently than wild-type virus in dividing cells. The conservation of the thymidine kinase gene ensures that the mutant remains sensitive to antiviral drugs such as acyclovir, which are activated by the viral thymidine kinase. In R849, both copies of the $\gamma_1$34.5 gene have been replaced by the *E. coli LacZ* reporter gene.

Herein, we report that vein grafts infected *in vitro* and *in vivo* with the genetically engineered HSV-1 exhibit stable expression of reporter genes in the SMC layer. Gene expression persists for up to 4 weeks after infection and can be abrogated following acyclovir administration. Experimental vein grafts treated with R849 exhibit markedly decreased SMC proliferation and restenosis suggesting the potential utility of HSV-1 vectors in the treatment of neointimal hyperplasia.

Results

Transfection of human saphenous veins in whole organ culture

To explore the feasibility of infecting human vascular tissue with the HSV-1 vector, and to examine the effect of HSV-1 on the integrity of the endothelium, human saphenous vein segments discarded after coronary or peripheral bypass were procured as previously described.[9] The segments were 3.5–5.5 mm in diameter and were judged to be of good quality by the operating surgeon. No patient had any history of venous disease. Segments were exposed to R849 approximately 1×10^9 plaque forming units (p.f.u.) for 10 min at 120 mm Hg and then cultured whole for 0–55 days. All veins exposed to R849 demonstrated significant β-galactosidase expression in the intima and adventitia and, to a lesser extent, the media (Figure 2a). Expression persisted to day 55, the final time point of the experiment. β-Galactosidase staining was not observed in vein segments exposed to vehicle only (phosphate buffered saline, PBS). Importantly, R849 treatment did not appreciably affect the integrity of the endothelial layer as assessed qualitatively by the presence of von Willebrand factor, a human endothelial cell marker (Figure 2b).

Infection with HSV-1 is stable and efficient in vivo

HSV-1 gene transfer was tested in the New Zealand White rabbit carotid vein patch model. Surgically isolated external jugular veins were exposed to R849, R3616, or viral medium for 10 min at 110–120 mm Hg, and then sutured above the open common carotid artery (vein patch angioplasty). This model effectively mimics the hemodynamic conditions of arterial transplantation but is technically simpler than interposition grafting.[19] All operations were completed without complication. There was no significant difference in mean blood flow in vehicle-treated (31 ± 2 ml/min: $n = 6$) *versus* viral-transfected patches (34 ± 3 ml/min; $n = 10$; $P = 0.3$) at the time of implantation. Also, there were no differences in serum electrolytes or renal function throughout the 4-week study period. All vein patches were patent at the time of harvest at 4 weeks ($n = 16$). Patches exposed to R849 showed marked β-galactosidase staining in all parts of the vessel wall, most notably within the medial layer (Figure 3). Calculated infection efficiencies (percent of cells infected) were $42 \pm 2\%$ in the adventitia, $44 \pm 9\%$ in the media, and $45 \pm 6\%$ in the neointima. Moderate β-galactosidase staining was also detected in the untreated arterial segment adjacent to the vein patch, and in the more distal portions of the native carotid artery.

Treatment with acyclovir eradicates viral infection

In a second series of experiments, rabbits with vein patches infected with R849 were treated with acyclovir

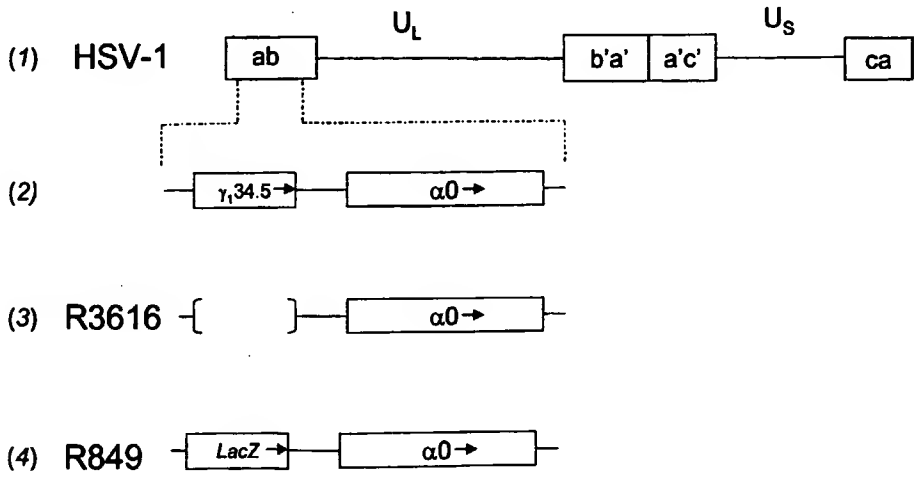

Figure 1 Schematic representations of DNA sequence of the HSV-1 vectors. Line 1, sequence arrangement of HSV-1 genome (>152 kb). The HSV-1 genome consists of two unique sequences represented as lines, long (UL) and short (US) flanked by inverted repeat sequences ab and b'a' and a'c' and ca, respectively represented as rectangles. Line 2, expansion of the ab repeat showing the position of the $\gamma_1$34.5 gene in relation to other genes mapping in the repeat. Line 3, schematic representation of the corresponding sequence in R3616 virus which lacks the $\gamma_1$34.5 genes. Line 4, schematic representation of the corresponding sequence in the R849 virus in which the LacZ gene was inserted in place of the $\gamma_1$34.5 gene.

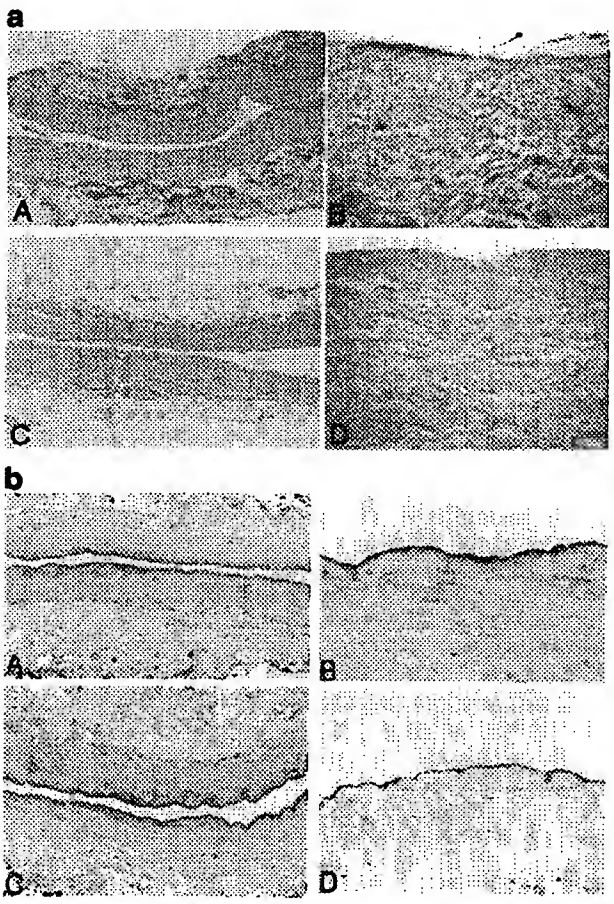

Figure 2 (a) HSV-1-mediated reporter gene expression in cultured human saphenous veins. Panels (A) and (B) show human saphenous vein 34 days after exposure to R849 (magnification 4× and 40×, respectively). Blue staining shows the presence of β-galactosidase reporter transgene expression. Panels (C) and (D) show human saphenous vein 34 days after exposure to vehicle only (magnification 4× and 40×, respectively). (b) HSV-1-exposed human saphenous vein endothelial integrity as assessed by von Willebrand factor (vWF) immunohistochemistry. Panels (A) and (C) show cultured human veins 34 days after exposure to vehicle and R849, respectively (magnification 5×). The presence of vWF in intact endothelial cells is marked by the brown stain. Panels (B) and (D) show the same segments at 40× magnification.

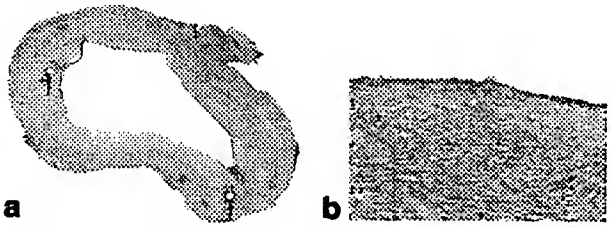

Figure 3 HSV-1-mediated marker gene transfer in rabbit external jugular vein patches. Panels (a) and (b) show an R849-exposed vein patch harvested after 4 weeks at 4× and 40× magnification, respectively. The superior portion of the reconstruction is the vein patch and the inferior portion is the common carotid artery. Arrows denote suture artifact separating the artery and vein. Reporter gene expression is localized by blue staining. There was no detectable staining in any of the R3616- or vehicle-treated subjects. There was no appreciable systemic toxicity of viral treatment as measured by serum electrolyte or creatinine concentrations (data not shown).

to assess its ability to eradicate the established viral infection. Acyclovir therapy was begun 1 week postoperatively with maintenance of serum levels within the therapeutic range (9 ± 4 μg/ml; $n = 6$). After 4 weeks, vein patches were excised and stained with X-gal. Reporter gene expression was almost completely eliminated, with β-galactosidase activity evident in <1% of cells when compared with the untreated R849 controls ($P < 0.005$; Figure 4). There was no significant systemic toxicity from acyclovir treatment as measured by serum electrolytes, blood urea nitrogen and creatinine levels throughout the study.

Treatment with HSV-1 significantly reduces SMC proliferation and restenosis

A final series of experiments was performed to test the effect of HSV-1 treatment on pathological restenosis. Rabbit external jugular veins were treated with R849 or viral culture medium and then fashioned as vein patches as described above. The common carotid artery was ligated distal to the cranial thyroid artery in order to increase outflow resistance, decrease shear stress, and stimulate restenosis.[19] This model was used to simulate a failing bypass graft and consistently reduces blood flow to <10% of baseline levels. After 4 weeks, the patches were re-exposed, perfusion-fixed with glutaraldehyde, and examined for restenosis. R849-infected patches exhibited

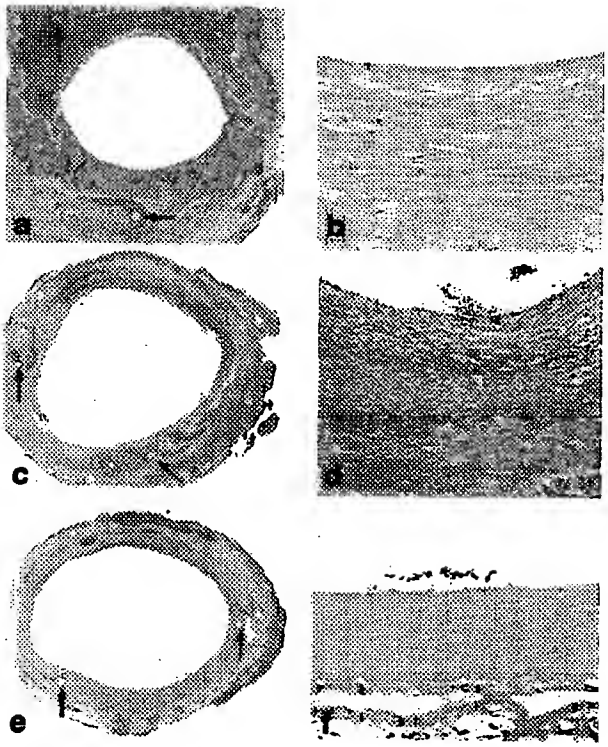

Figure 4 Suppression of HSV-1 infection of vein patches with acyclovir. Panels (a and b) show low flow vehicle-infected control, (c and d) low-flow R849-infected patch, (e and f) low-flow R849-infected patch + acyclovir. The superior half of each low power section is the external jugular vein patch, the inferior half is the underlying common carotid artery (a, c, e; magnification 4×). The arrow denotes suture artifact between the artery and vein. Note the blue staining in the R849-treated patch (c, d) which is almost completely eradicated by acyclovir treatment (e, f). In the right panels (b, d, f; magnification 40×) are examples of the vein patch.

significantly less neointimal thickening (280 ± 28 μm; $n = 8$) than vehicle-treated controls (410 ± 38 μm; $n = 6$; $P = 0.03$), so much so that the R849-treated 'low-flow' patches morphologically resembled the 'high-flow' controls (Figure 5).

Discussion

The present studies demonstrate that HSV-1 attenuated mutant virus stably infects all three layers of the vascular wall without deliberate preliminary injury to the endothelium. The amount of virus required (approximately 10^9 p.f.u.) is 1000-fold lower than the average dose of adenovirus vector required for a similar level of infection.[20] Reporter gene expression is consistently detected for 55 days in cultured human veins and for 4 weeks in immunocompetent animals. This is the longest duration of gene expression that has been reported to date in vascular tissue. Reporter gene expression was arrested following administration of acyclovir potentially offering a level of control not possible with any other vector used for this purpose. Lastly, a reduction in SMC proliferation and experimental restenosis was observed, essentially returning a 'low-flow' vascular circuit to that of 'high-flow' morphology.

The drawbacks of adenoviral gene therapy for vascular disease and the high infectivity of HSV-1 stimulated the current study. Genetically modified HSV-1 has been extensively tested as potential therapy for malignant tumors of the central nervous system.[16–18] Initial protocols called for viruses that lacked one or more genes, such as thymidine kinase or ribonucleotide reductase, which reduced viral growth in non-dividing cells.[21–23] A second strategy, and the one proposed herein, centers on the deletion of both copies of the $\gamma_1$34.5 gene.[24,25] Deletion of both copies potentially decreases virulence, but still allows for replication in dividing cells. In addition, the thymidine kinase (TK) gene is maintained. TK converts pro-drugs ganciclovir or acyclovir into a toxic nucleotide analogue, the incorporation of which blocks viral replication. Retention of the TK gene, therefore, allows for potential eradication of established viral infection and pharmacologic control of transgene expression.

The hypothesis that $\gamma_1$34.5-deleted HSV-1 would be an ideal vector for vascular gene transfer was examined in this study. The results showed that, in animals having an intact immune system, the vector was capable of maintaining reporter gene expression in 45% of arterialized venous neointimal cells for up to 4 weeks. This duration and efficiency far exceeds that reported with other vectors.[9,11,20,26–29] Furthermore, the dose of HSV-1 required to achieve prolonged gene expression (approximately 10^9 p.f.u./ml) was several orders of magnitude less than traditional strategies using adenovirus.[8,9,13,20,27–38] Although the safety profile of the current vector is largely unknown, no toxicity or immunostimulation was detected using this dosing regimen.

Perhaps the most important finding in this study was the ability of acyclovir to arrest HSV-1 replication in infected tissues. HSV-1 TK is the most widely used suicide agent for cancer gene therapy. Tumor cells that express HSV-1 TK are rendered sensitive to pro-drugs, such as acyclovir, due to preferential phosphorylation. This paradigm has been used in multiple antitumor protocols,[22,39–41] and was further tested in the present study. A standard treatment regimen of acyclovir successfully eradicated reporter gene expression from infected vascular tissue. This level of control has obvious implications for the safety profile of gene therapy.

In summary, these experiments suggest that prevention of restenosis using the HSV-1 vector is feasible. The results obtained from the *in vivo* model system with an 'off-the-shelf' recombinant virus may eventually lead to the development of vectors specifically designed for the prevention of vein graft failure, but which still retain the capacity to respond to antiviral drugs for customized extent and duration of viral gene expression.

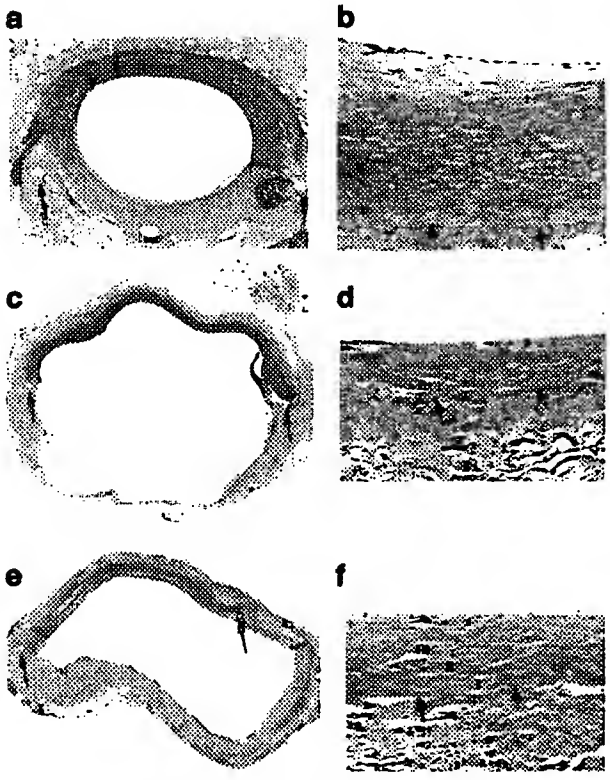

Figure 5 HSV-1-mediated prevention of restenosis in rabbit vein patches. (a and b) Low-flow vehicle-infected vein patch, (c and d) high-flow vehicle-infected vein patch (historical control), (e and f) low-flow R849-infected patch. In the left panels (a, c, e; magnification 4×), the superior portion of the reconstruction is the the common carotid artery, the inferior portion is the external jugular vein patch, and the arrow marks the intervening suture artifact. In the right panels (b, d, f; magnification 40×), the arrows denote the level of the internal elastic lamina.

Materials and methods

Preparation of viral vectors

R3616 and R849 viruses were prepared as previously described.[18,24]

Transfection of human saphenous veins in whole organ culture

Human saphenous veins were transfected and cultured whole as previously described.[9] Briefly, segments of saphenous vein were harvested from patients at the time of coronary or peripheral bypass surgery under an approved protocol. Segments were gently irrigated with

saline to remove any residual debris, and excess adventitia trimmed sharply. The vein was divided into control and experimental segments and separately dually cannulated for vector infusion and pressure monitoring (Hewlett-Packard 78534B; Palo Alto, CA, USA). Veins were distended with either vehicle (n = 4) or R849 6.5 × 10^8 p.f.u./ml (n = 4; 1.0–6.0 ml) for 10 min at 120 mm Hg. The main channel of the vein was then rinsed with saline and the vein cut into rings 0.5 cm in length. The first ring was fixed immediately in 1.25% glutaraldehyde for 10 min, washed in PBS, and incubated overnight at 37°C with X-gal (GibcoBRL, Rockville, MD, USA) reagent (5-bromo-4-chloro-3-indoyl-b-D-galactosidase) in a dark environment. The remaining rings (between four and seven depending on amount of vein available) were each placed in separate culture wells with 6 ml media (RPMI-1640 with 15% FBS; BioWhittaker/Clonetics, Walkersville, MD, USA) and incubated at 37°C. Media was changed every 2 days. Vein segments were removed from the medium, fixed, and stained with X-gal as above after 3, 5, 10, 14, 20, 28, 32, 34, 40, 48 and 55 days.

Sections were also stained with an antibody to von Willebrand factor (vWF; Dako, Glostrup, Denmark) to examine the integrity of the endothelium. Four-μm sections of paraffin-embedded tissues were mounted on poly-L-lysine coated slides, and deparaffinized by sequential 5 min washes with xylene and decreasing concentrations of EtOH and dH_2O. Sections were then incubated in pre-warmed 10 mM EDTA (pH 6.0) for 60 min at 70°C, washed three times with dH_2O, and incubated in pre-warmed Proteinase K (20 μg/ml in PBS) for 20 min at 37°C. After blocking with a 10% solution of normal goat serum (Vector, Burlingame, CA, USA) in 0.5% casein (Sigma, St Louis, MO, USA) and PBS, endogenous avidin and biotin were quenched using a blocking kit (Vector). Slides were washed three times with 0.5% casein/0.01% Tween 20 in PBS and incubated overnight at 4°C with rabbit anti-human vWF (Dako) titered to 0.8 μg/ml in 0.5% casein. Appropriate negative controls (rabbit IgG isotype, Dako) were also used. Slides were washed three times and incubated with goat-anti-rabbit IgG biotinylated secondary antibody (2 μg/ml in PBS, 0.5% casein and 10% normal human serum) for 25 min at 37°C. Endogenous peroxidase was quenched with 3% H_2O_2 (20 min at RT), and slides were washed and incubated with Vectastain Standard Elite ABC kit (Vector) for 25 min at RT. Tissues were developed with the Vector DAB kit, and counterstained with a 0.03% solution of light green SF yellowish (Fisher, Hanover Park, IL, USA). Sections were dehydrated in alcohol and xylene, mounted with Permount and coverslips, and examined under light microscopy.

Transfection of rabbit external jugular vein patches

Animals were cared for in accordance with the University of Chicago Institutional Animal Care and Use Committee (IACUC). Male New Zealand White rabbits (3.5–4 kg) were anesthetized by intramuscular injection with ketamine hydrochloride (40 mg/kg) and xylazine (5 mg/kg) augmented with halothane via endotracheal intubation. Antibiotic prophylaxis was provided with enrofloxacin 10 mg/kg i.m. daily. Using sterile technique, the external jugular vein was exposed and two branches cannulated with 24-gauge catheters. One cannula was used for irrigation and transfection, and the other for intraluminal pressure monitoring. The main channel was transfected with either vehicle (n = 6), R849 4 × 10^8 p.f.u./ml (n = 8; 0.2–4.0 ml) or R3616 3 × 10^8 p.f.u./ml (n = 2; 1.0–2.0 ml) for 10 min at 100 mm Hg. Following transfection, the vein was irrigated with saline, excised and bivalved. The ipsilateral common carotid artery (CCA) was then exposed through the same incision and the animal systemically anticoagulated with heparin 200 U/kg intravenously. The CCA was doubly clamped and a 1.5-cm longitudinal arteriotomy made proximal to the cranial thyroid branch. The arteriotomy was reconstructed with external jugular vein patch angioplasty using running 8-0 polypropylene suture (Davis-Geck TE-145; Manatee, Puerto Rico) under 4.5x-loupe magnification. Ultrasonic transit-time flow through the graft was measured (Transonics Systems Inc. HT207; Ithaca, NY, USA) and recorded using a digital data acquisition system (Lab Master DMA; Scientific Solutions, Solon, OH, USA). The incision was closed and the animal allowed to recover. Anticoagulation was not reversed. Following 1 week of recovery, half the rabbits infected with R849 (n = 4) or exposed to vehicle (n = 2) were cannulated with a 24-gauge i.v. and maintained for a total of 5 days on intravenous acyclovir (Abbott Laboratories, North Chicago, IL, USA; 75 mg/kg bodyweight) in a divided dose. Starting on the same post-operative day, the rabbits were simultaneously treated with oral acyclovir (Alphaparm USPD, Carole Park, Australia; 3 mg/ccH_2O) which was maintained until death. Baseline acyclovir, serum electrolyte, blood urea nitrogen, and creatinine levels were measured before initiation of acyclovir treatment and were repeated on days 2, 5 and 10 after initiation of treatment, and at death. After 4 weeks, the animals were re-anesthetized and the vein patches re-exposed. Intra-arterial pressure and blood flow through the patch were again measured and recorded. The vessel was perfusion fixed with 1.25% glutaraldehyde for 10 min at 120 mm Hg and the vein patch divided into three sections starting 1 mm distal to the beginning of the patch and ending 1 mm proximal to its endpoint. The harvested vessels were fixed in 1.25% glutaraldehyde for 10 min, washed in PBS, and incubated overnight at 37°C with X-gal. Five-μm sections were cut from each of the portions of the patch and stained using eosin, hematoxylin and eosin (H&E) and the Weigert van Gieson method. Ten high-power fields (40×) of each tissue section were examined using a microscope (Nikon Microphoto-FX, Tokyo, Japan) equipped with a digital camera (Sony PowerHAD 3CCD, Park Ridge, NJ, USA). Stained cells/nuclei were counted using standard imaging software (ImagePro Plus 3.0.1; Media Cybernetics, Silver Springs, MD, USA). The number of β-galactosidase-positive cells were counted in the neointima, media and adventitia located circumferentially about the lumen. The total number of cells were counted on the H&E sections in the neointima, media and adventitia. The average number of positive cells was expressed as a percentage of the total number of cells and considered to be the transfection efficiency. Sections stained using the Weigert van Gieson technique were analyzed using digital planimetry (Adobe Photoshop 5.0; Adobe Systems, San Jose, CA, USA). Neointimal hyperplasia, defined as the distance between the internal elastic lamina and lumen, was measured at 10 points evenly spaced over the vein patch. The area encompassed by the internal elastic lamina, as well as the lumen area were

measured and their ratio used to calculate area and diameter stenosis.

Statistics

Statistical analysis was performed using SPSS 8.0 software (SPSS, Chicago, IL, USA). Comparison of means was studied using a Student's t test. Significance was assigned at $P < 0.05$.

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